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review

A topological view of the replicon

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The replication of circular DNA faces topological obstacles that need to be overcome to allow the complete duplication and separation of newly replicated molecules. Small bacterial plasmids provide a perfect model system to study the interplay between DNA helicases, polymerases, topoisomerases and the overall architecture of partially replicated molecules. Recent studies have shown that partially replicated circular molecules have an amazing ability to form various types of structures (supercoils, precatenanes, knots and catenanes) that help to accommodate the dynamic interplay between duplex unwinding at the replication fork and DNA unlinking by topoisomerases.

Keywords: replication; plasmid; supercoiling; catenane; knot

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Introduction

The replicon model was the first attempt to explain how DNA replication is regulated in bacteria (Jacob *et al*, 1963). Originally formulated on the basis of observations made in *Escherichia coli*, it was later extended to plasmids, phages and the chromosomes of all prokaryotes and eukaryotes. Forty years later, the key aspects of the replicon model still hold true and during this time it has inspired numerous significant discoveries (Jacob, 1993; Nordstrom, 2003). Briefly, the model developed the theme of the units of replication, which the authors called replicons. The regulation of DNA replication was claimed to involve at least two elements: a specific protein, the initiator, and a target DNA sequence, the replicator, nowadays known as the 'origin of replication'. Just a couple of years after François Jacob, Sydney Brenner and François Cuzin launched the replicon model at a meeting in Cold Spring Harbor (Jacob *et al*, 1963), Gerome Vinograd and co-workers found that the circular genome of the polyoma virus is supercoiled (Vinograd *et al*, 1965). This observation was later extended to virtually all circular duplex DNA (Cozzarelli, 1980). Supercoiling, which literally means coiling of a coil, is a topological property of DNA molecules in which the double helix twists around its own axis in three-dimensional space (Bowater, 2002). The finding that DNA is supercoiled, together with the discovery of topoisomerases (Wang, 1971) opened a whole new field in molecular biology: DNA topology (Wang, 2002).

The principal aim of this review is to summarize what is known about the topological changes that take place as a replicon replicates. We focus on small bacterial plasmids, as most of the studies that have addressed this issue have used pBR322 and other small derivatives as a model system. It should be noted, however, that it is not always feasible to extrapolate the observations made on small plasmids to bacterial or eukaryotic chromosomes. Plasmids are small topological domains that do not necessarily reflect the conditions of the large domains of the chromosomes of prokaryotes and eukaryotes (Higgins &Vologodskii, 2004).

Primer on DNA topology and DNA topoisomerases

A DNA molecule is said to be negatively (–) supercoiled when the linking number (the minimal number of passages needed of one strand through another to separate them) is lower than in the relaxed circular DNA of the corresponding size. Both *in vivo* (Bliska & Cozzarelli, 1987) and *in vitro* (Bednar *et al*, 1994), (–) supercoiled bacterial plasmids are known to adopt a right-handed intertwined configuration in which the duplex–duplex crossings have a (–) sign (see Fig 1A,B for an explanation). In eukaryotic cells, (–) supercoiling is constrained by the left-handed winding of the DNA around nucleosomes, resulting in a toroidal winding in which duplex–duplex crossings also have a (–) sign (Fig 2B). As shown in Fig 1A, local strand separation by a DNA helicase in (–) supercoiled DNA molecules initially leads to the relaxation of (–) supercoiling, whereas further separation causes the accumulation of positive (+) supercoiling. The arising torsional stress opposes further helicase action and topoisomerases are required for further separation of the DNA strands.

Topoisomerases are enzymes that interconvert different topological states of DNA. They are divided into type I and type II enzymes, which transiently cleave one or both strands of DNA, respectively. Type I topoisomerases are additionally divided into two subtypes: A and B. Enzymes belonging to the subtype A have a complex mechanism of action that involves passage of the uncut strand through the enzyme-bridged cleavage of the other strand. Interestingly, while acting on DNA with nicks or with single-stranded regions, type IA topoisomerases can cleave the continuous strand and allow the passage of a segment of duplex DNA of the same or another DNA molecule through the cut strand. Topoisomerases of the subtype IB act by a simpler mechanism that involves free rotation of DNA at the transient nick site (Stasiak, 2003). There are two type I topoisomerases in *E. coli* that are known as topo I and III and they both belong to subtype A (Champoux, 2001). Importantly, *E. coli* topo I and III are hardly

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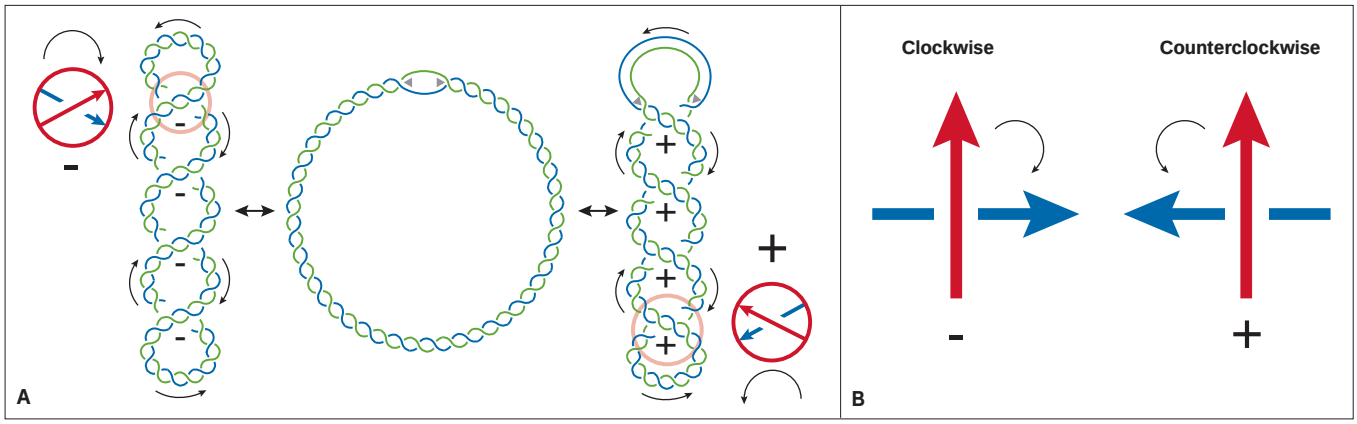

Fig 1 | Supercoiling: its handedness and sign. (**A**) Negatively supercoiled DNA (left) loses supercoiling due to local DNA unwinding mediated by DNA helicases (shown as grey wedges) and then becomes (+) supercoiled by further strand separation. Notice that the intertwined superhelix is right-handed in (–) supercoiled molecules and left-handed in (+) supercoiled ones. The sign of the duplex–duplex crossings (see panel **B**) changes from (–) to (+) upon a change from negative to positive supercoiling. (**B**) Topological convention of sign assignment of perceived crossings. In a (–) crossing, one would need to turn the overlying direction arrow clockwise to align it with the underlying direction arrow (the rotation needs to be smaller than 180°). In a (+) crossing the required rotation would be counterclockwise. Notice that orientation of the underlying and overlying direction arrows at each crossing are not independent from each other but result from assigning a consistent direction along the whole DNA molecule analysed. To facilitate sign recognition in **A** and **B**, the overlying and underlying direction arrows are marked in red and blue, respectively.

active on the bulk of cellular DNA that is maintained at physiological levels of (–) supercoiling. Non-physiologically strong (–) supercoiling or the presence of single-stranded regions activate topo I and III (Champoux, 2001). Type II topoisomerases make transient double-stranded breaks and allow the passage of another duplex across the break. They are usually ATP-dependent (Gellert *et al*, 1976). There are two type II topoisomerases in *E. coli,* which are known as DNA gyrase and topo IV (Champoux, 2001). As with *E. coli* type I topoisomerases, gyrase and topo IV are also hardly active on the bulk of cellular DNA and become activated by DNA relaxation in the case of gyrase and by (+) supercoiling in the case of topo IV. It is important for energy balance that there is no futile action of topoisomerases on the bulk of DNA through which a gyrase, for example, would continuously use ATP to introduce (–) supercoiling and topo I or III would relax the DNA. Topoisomerase action therefore needs to be limited to the biological processes that involve DNA, such as replication, transcription, recombination and repair during which DNA topology needs to be modified.

Tug-of-war between (–) and (+) supercoiling

To initiate their replication, bacterial plasmids must be (–) supercoiled as this facilitates strand separation at the origin of replication (Fig 1A; Funnell *et al*, 1987; Marians *et al*, 1986). Once initiation has been accomplished, elongation proceeds by means of a complex ensemble of enzymes known as the replisome. The current view is that during replication, DNA passes through a stationary replisome. In front of this replisome, a hexameric DNA helicase separates the parental strands that are to be used as templates. This strand separation leads to overwinding (positive supercoiling) of the duplex ahead of the fork (Fig 1A; Alexandrov *et al*, 1999; Peter *et al*, 1998; Ullsperger *et al*, 1995). However, (–) supercoiling is important for the opening of the DNA double helix (Crisona *et al*, 2000; Kanaar & Cozzarelli, 1992). How then do replication intermediates (RIs) manage to remain (–) supercoiled as the fork advances? The first clue to answer this question came with the discovery of DNA gyrase (Gellert *et al*, 1976). It is thought that the continuous action of gyrase on the unreplicated portion of replicating plasmids decreases the linking number of the parental duplex (Alexandrov *et al*, 1999; Peter *et al*, 1998; Ullsperger *et al*, 1995). In this way, gyrase helps to compensate for the overwinding of the duplex as the fork advances. The rate of unlinking by gyrase, however, is slow and might be insufficient to sustain the rate of fork movement in *E. coli* (Peter *et al*, 1998). Furthermore, DNA gyrase can actively cause unlinking only when acting on the unreplicated portion of replicating plasmids (Gellert *et al*, 1976; Kampranis *et al*, 1999). At early stages of replication, when the unreplicating portion is sufficiently long, several gyrase molecules could work in parallel to sustain a high speed of unlinking. As the length of the unreplicated portion shrinks, however, there is less space for gyrase to act. Each gyrase molecule needs around 150 base pairs to bind to DNA (Bates & Maxwell, 1989), and so overwinding caused by the progressing fork may eventually accumulate. This potential problem was first recognized by James Champoux and Michael Been (Champoux & Been, 1980), who realized that this gyrase deficit would eventually lead to the accumulation of (+) supercoiling at later stages of the replication process. To solve this dilemma, they proposed that supercoiling might diffuse throughout the replication fork and redistribute both ahead of and behind the fork. In this model, the other type II topoisomerase, topo IV, which is the main decatenase in *E. coli* (Zechiedrich & Cozzarelli, 1995; Zechiedrich *et al*, 1997), assists gyrase to compensate for the overwinding that accumulates as the fork advances. Brian Peter and coworkers (Peter *et al*, 1998) used electron microscopy to confirm the diffusion of supercoiling across the fork in an *in vitro* assay that yielded partially replicated plasmids containing stalled forks. They called the intertwining of the sister duplexes in the replicated portion "precatenanes" to distinguish them from the supercoiling in the unreplicated portion (Figs 2A and 3B,D,E). The emerging idea was that unlinking of the parental duplex during DNA replication is carried out by gyrase introducing (–) supercoils ahead of the fork and topo IV removing precatenanes behind the fork. This would explain why progression of the replication fork is impeded when both gyrase and topo IV are mutated

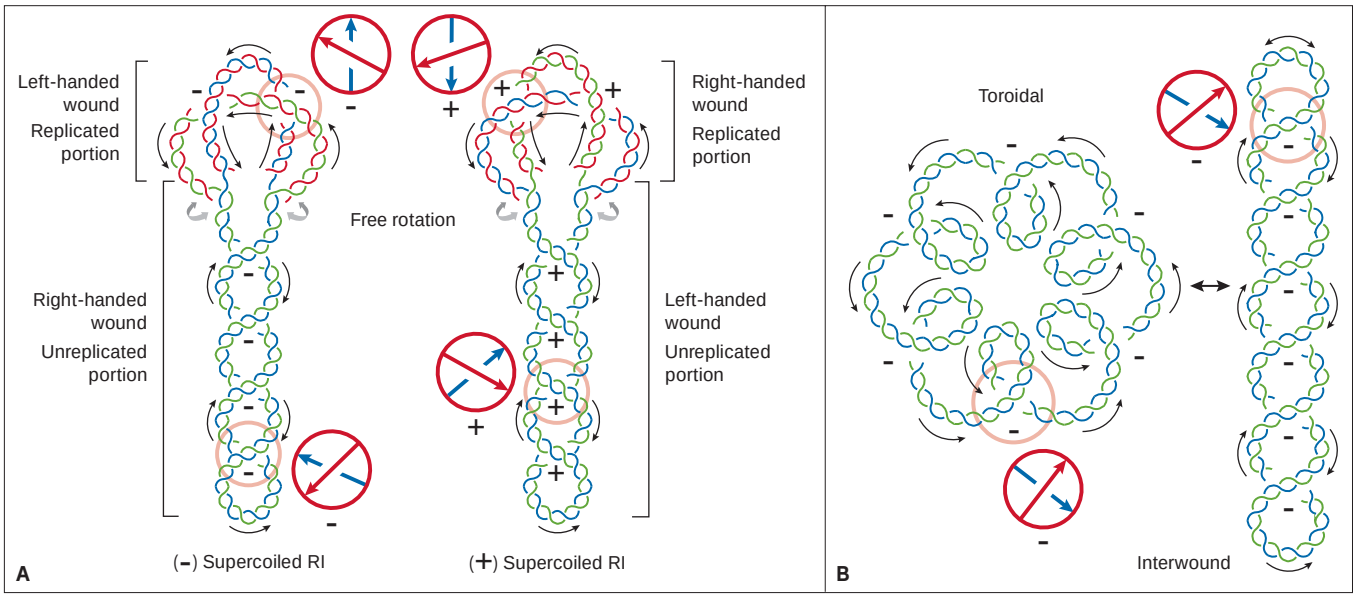

Fig 2 | Topological sign and handedness of duplex–duplex intertwining in supercoiled replication intermediates. (**A**) Schematic drawing of (–) and (+) supercoiled replication intermediates (RIs). (**B**) Elastic transition between toroidally wound (around core histones for example) and intertwined form of (–) supercoiled DNA. Notice that, in the toroidally wound form, the segments that cross in a projection run in the same direction around a virtual torus, whereas, in the intertwined form, the crossing segments run in opposite directions around the virtual cylinder enclosed by the DNA. This change of relative orientation causes the topological signs to remain the same despite a perceived change from left- to right-handed winding of the superhelices. The mathematical convention applied in DNA topology assigns a parallel orientation to both strands of DNA (this is required to have a (+) linking number in B-DNA, which forms a right-handed helix; Bates & Maxwell, 1993). For this reason, to trace the linking number contribution of parental strands in an RI, one needs to assign the same direction to both newly replicated duplex regions. In (–) supercoiled DNA there is a tendency to release the torsional stress by left-handed winding of unpaired strands or by flipping runs of alternating purine–pyrimidine from right-handed B-DNA to the left-handed Z-DNA (DiCapua *et al*, 1983). It is therefore energetically favourable in deproteinized (–) supercoiled RIs that the newly synthesized duplex regions are wound around each other in a left-handed way. The opposite situation applies to (+) supercoiled RIs. The parental duplex is indicated in blue and green, whereas nascent strands are depicted in red.

or inhibited (Hiasa *et al*, 1994; Khodursky *et al*, 2000; Levine *et al*, 1998). It is important to notice that for supercoiling to diffuse across the replication fork, the sister duplexes should be able to rotate freely around each other at the forks. Curiously, for a (–) supercoiled RI *in vitro*, the parental duplex winds around itself in a right-handed manner ahead of the fork, whereas behind the fork the sister duplexes wind in a left-handed manner (Postow *et al*, 2001a). The reverse occurs in the case of (+) supercoiled RIs (see Figs 2A and 3B,D,E). It is rather non-intuitive that the direction of intertwining of opposing double-stranded regions changes between the unreplicated and replicated portions of an RI that is under torsional stress. However, it is well known that an elastic transition from toroidal to intertwined forms of supercoiling changes the perceived handedness of intertwining while maintaining the same topological sign (Fig 2B; Bauer *et al*, 1980). Similarly, although the perceived handedness of duplex–duplex intertwining in replicated and unreplicated portions of supercoiled RIs are different, the topological sign of these crossings in both parts remain the same (Fig 2A).

As mentioned previously, (–) supercoiling assists any process that requires opening of the double helix (Crisona *et al*, 2000; Kanaar & Cozzarelli, 1992). Moreover, it was recently shown that for partially replicated molecules containing stalled forks, the introduction of net (+) supercoiling *in vitro* leads to replication fork reversal through the formation of a branched four-way Holliday-like junction, the so-called 'chicken-foot' structure (Olavarrieta *et al*, 2002c; Postow *et al*, 2001b; Sogo *et al*, 2002; Viguera *et al*, 2000). In short, it is thought that the coordinated action of gyrase and topo IV would allow RIs to remain (–) supercoiled throughout the replication process. Therefore, at any given time during replication, the degree of supercoiling would be the result of the balance between the action of at least the three different enzymes already mentioned: DNA helicase, leading to the accumulation of (+) supercoiling ahead of the fork; DNA gyrase, which introduces (–) supercoiling in the unreplicated portion; and topo IV, removing precatenanes behind the fork (Peter *et al*, 1998; Postow *et al*, 1999, 2001a).

Two-dimensional (2D) agarose gel electrophoresis of intact molecules formed *in vivo* with the fork stalled at different distances from the origin indicated that those plasmids with the fork stalled closer to the origin are more supercoiled than those with the fork stalled at increasing distances (Olavarrieta *et al*, 2002c). This observation suggests that although RIs remain (–) supercoiled throughout replication, they progressively relax as the fork advances. These results, however, should be examined with caution, as they do not necessarily reflect the situation during unimpaired DNA replication. In those plasmids containing stalled forks, there might be an excess of (–) stress due to the continuous action of gyrase once the fork has stalled.

Knotted bubbles as reporters of DNA topology *in vivo*

As soon as DNA topoisomerases were discovered, it was realized that DNA knots could form in living cells. Experimental evidence for knotted molecules *in vivo*, however, was scarce (Liu *et al*, 1981; Shishido *et al*, 1989; Shishido *et al*, 1987). It was therefore surprising

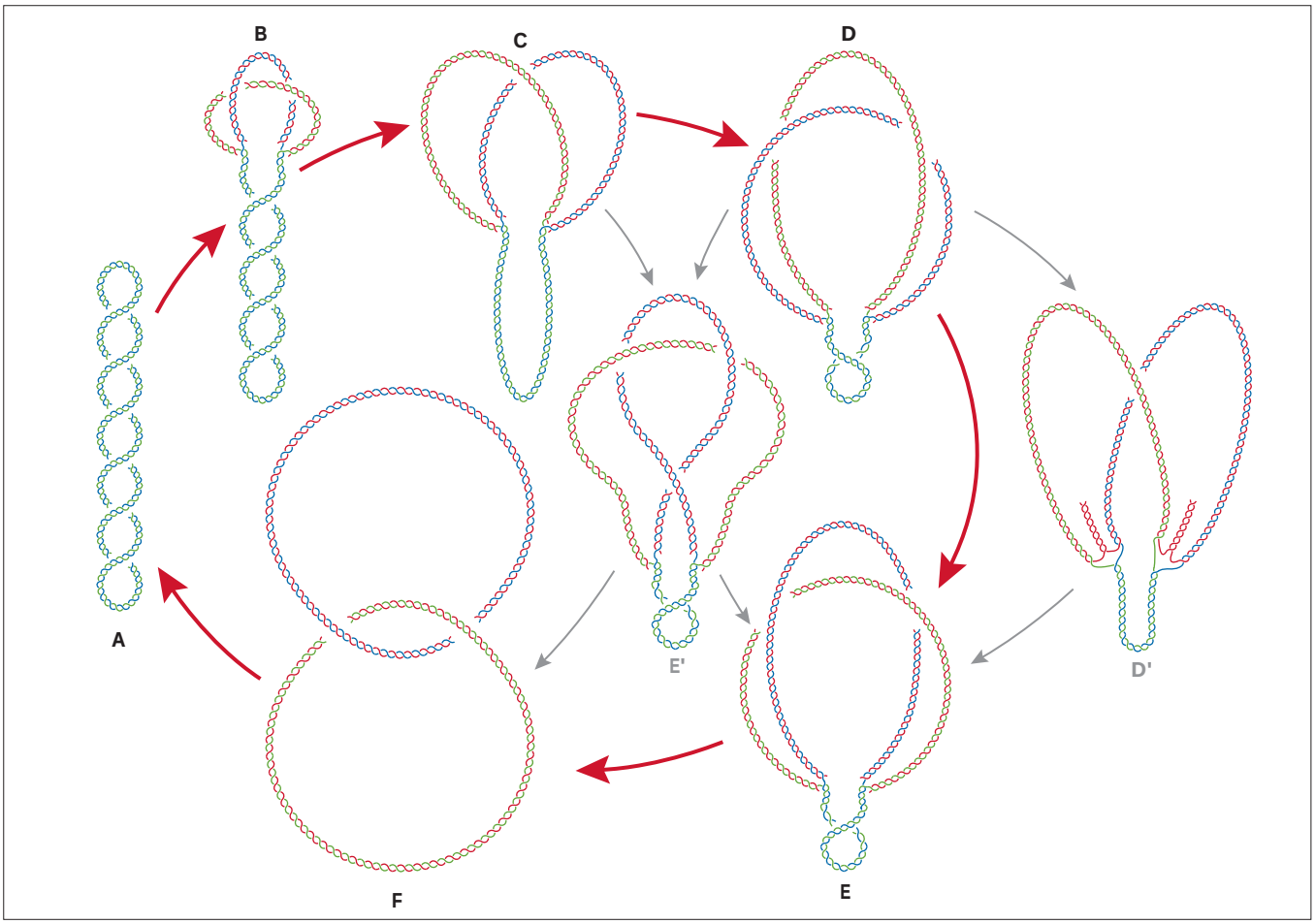

Fig 3 | The topological cycle of a replicon. (**A**) Unreplicated (–) supercoiled plasmid. (**B**) Twenty-five per cent replicated (–) supercoiled RI where the parental duplex winds right-handed, whereas the sister duplexes wind in a left-handed manner. (**C**) Fifty per cent replicated RI where supercoiling is zero. (**D**) Seventy-five per cent replicated (+) supercoiled RI where the parental duplex winds left-handed, whereas the sister duplexes wind in a right-handed manner. (**E**) Seventy-five per cent replicated (–) supercoiled RI where the parental duplex winds right-handed, whereas the sister duplexes wind in a left-handed manner. (**F**) One hundred per cent replicated catenane. (**E'**) Seventy-five per cent replicated (–) supercoiled RI bearing a knotted replication bubble. (**D'**) Seventy-five per cent replicated RI where supercoiling is zero containing two branched four-way Holliday-like junctions, called 'chicken-foot' structures. Red arrows indicate the putative most frequent pathway. Grey arrows show alternative pathways. The parental duplex is indicated in blue and green, whereas nascent strands are depicted in red.

when studies of bacterial plasmids with stalled forks revealed that such plasmids could be knotted *in vivo* and that they form a characteristic 'beads-on-a-string' arrangement of DNA bands in 2D gels (Santamaría *et al*, 1998, 2000; Viguera *et al*, 1996). The strategy used to identify these knotted bubbles involved cleavage in the unreplicated portion of the plasmids and resulted in the identification of knots confined within the replication bubbles. The characterization of the handedness of these knotted replication bubbles by electron microscopy (Sogo *et al*, 1999) indicated that the partially replicated molecules were (–) supercoiled when the knotting occurred (Postow *et al*, 1999).

Analyses of knotted replication bubbles in partially replicated molecules with the fork stalled at different distances from the origin indicated that the number and complexity of knotted replication bubbles increases as the fork advances (Olavarrieta *et al*, 2002b). It could be argued that the probability of knotting increases with bubble size. It is not that simple, however, as bubbles of the same size show more knots in small plasmids in which the fork stalls towards the end of replication (Olavarrieta *et al*, 2002c) compared with large plasmids in which the fork stalls at the beginning of the process (Olavarrieta *et al*, 2002b). Altogether these observations suggest that the probability of knotting behind the fork is inversely related to the precatenane's density (Fig 3B–E'). For regularly wound precatenanes, duplex–duplex passages are unlikely to 'trap' another segment of the same molecule, whereas this is not the case for loosely wound precatenanes (Sogo *et al*, 1999).

Once replication is completed, the remaining precatenanes and knotted replication bubbles automatically become catenanes (Fig 3F) that are eliminated by topo IV to allow segregation of the newly made sister duplexes (Lucas *et al*, 2001; Zechiedrich & Cozzarelli, 1995; Zechiedrich *et al*, 1997). It should be noted, however, that for the *E. coli* chromosome *in vivo* there might be alternative ways to decatenate sister duplexes (Ip *et al*, 2003). In any case, an increase in the number and complexity of knotted replication bubbles would increase the number of nodes in the catenane (the number of times each duplex winds around its sister) and this would be expected to have deleterious effects on the segregation of freshly replicated DNA

molecules. Leticia Olavarrieta and co-workers (Olavarrieta *et al*, 2002a) tested this hypothesis by comparing the number of knotted replication bubbles in plasmids in which the transcription of a selected gene and replication occur in the same or in opposite directions. The progression of transcription and replication in opposite directions is expected to drive the accumulation of (+) supercoiling between the forks as they approach each other (Brewer, 1988; Wu *et al*, 1988). The migration of this (+) supercoiling behind the fork would relax the regular intertwining of sister duplexes and lower the number of precatenanes. The number and complexity of knotted replication bubbles is indeed significantly higher when transcription and replication progress against each other (Olavarrieta *et al*, 2002a).

A zoo of replication intermediates

In summary, the current topological view of the replicon can be summarized as follows: circular plasmids need to be (–) supercoiled to initiate replication (Fig 3A). After initiation, supercoiling is distributed between the unreplicated and replicated portions (Fig 3B). RIs progressively relax as the fork advances and towards the end of replication, they could lose all native (–) supercoiling (Fig 3C). At later stages, they could even acquire net (+) supercoiling (Fig 3D) that would, however, be eliminated by the combined action of gyrase and topo IV to restore the native (–) supercoiling (Fig 3E). Finally, once replication is completed, all remaining precatenanes become catenanes (Fig 3F) and their decatenation by topo IV allows the two sister duplexes to segregate freely to complete the cycle. The probability of knotting behind the fork is inversely related to the precatenane's density. For this reason knotted bubbles can form, in particular towards the end of replication (Fig 3E'). The RIs bearing knotted replication bubbles could be either (–) or (+) supercoiled. These knotted bubbles could be unknotted by topo IV during replication or otherwise become catenanes once replication is completed. Alternatively, the transient accumulation of (+) supercoiling could lead to fork stalling and regression through the formation of the 'chicken-foot' structure. These transient intermediates could be rescued, however, by the combined action of topo IV and DNA gyrase to restore (–) supercoiling and reverse fork regression (Fig 3D').

The topo IV decatenation paradox

It was recently found that topo IV relaxes (+) supercoils at a 20-fold faster rate than (–) supercoils (Crisona *et al*, 2000). Furthermore, *in vitro* assays showed that topo IV recognizes the chiral crossings imposed by the left-handed superhelix of (+) supercoiled DNA (Charvin *et al*, 2003; Stone *et al*, 2003; Trigueros *et al*, 2004). This observation unmasked a new paradox. As previously stated (Figs 2A and 3B), for (–) supercoiled RIs *in vitro*, the parental duplex winds around itself in a right-handed manner ahead of the fork, whereas behind the fork the sister duplexes wind in a left-handed manner. If this situation also applies *in vivo*, left-handed precatenanes in (–) supercoiled RIs would be recognized and eliminated by topo IV in a preferential manner. In such a case, topo IV action would be detrimental, as it would eventually increase the linking number of the parental strands. Note that gyrase would be burning ATP to pump (–) supercoils ahead of the fork while topo IV would be burning more ATP to eliminate these very same (–) supercoils once they diffuse through the fork and become left-handed precatenanes. Moreover, the right-handed precatenanes present in (+) supercoiled RIs would not be eliminated by topo IV. These observations call into question the precatenane model. It is possible that in actively replicating molecules, supercoiling does not diffuse through the replication forks because they might not be able to rotate freely. The observation that replication complexes are anchored to the bacterial membrane (Levine *et al*, 1998) suggests that there is a topological barrier that would prevent diffusion of the (+) supercoiling generated in the unreplicated portion as the fork advances to the replicated part. In this case, precatenanes would not form. Experimental evidence for precatenanes *in vivo* is not abundant and the few cases reported in the literature are indirect. The occurrence of knotted replication bubbles (Olavarrieta *et al*, 2002a,b,c; Sogo *et al*, 1999; Viguera *et al*, 1996) was considered to be the best available evidence indicating that precatenanes may form in (–) supercoiled, partially replicated molecules *in vivo* (Postow *et al*, 1999). Further evidence supporting the occurrence of precatenanes *in vivo* comes from experiments using small circular plasmids replicated in *Xenopus* cell extracts. The significant increase in the number and complexity of catenanes after partial inhibition of eukaryotic topo II (the equivalent of prokaryotic topo IV) could only derive from pre-existing precatenanes (Lucas *et al*, 2001). In most of these cases, though, replication was impaired either by stalling the forks or by inhibiting topo II. It is possible that precatenanes could form *in vivo* only if progression of the replication forks is permanently stopped or severely impaired. In other words, precatenanes might not form *in vivo* during unimpaired DNA replication. They would readily form *in vitro*, however, after DNA isolation and deproteinization, as then the forks would be free to rotate. It is interesting to note that in *E. coli* cells, the majority of topo IV activity is concentrated close to replication factories at the cell centre and occurs mainly late in the cell cycle (Espeli *et al*, 2003; Sherratt, 2003). These findings could explain how topo IV is prevented from eliminating left-handed precatenanes in (–) supercoiled RIs if such precatenanes eventually form.

The topological changes that take place as a replicon replicates are just beginning to be unravelled. Until this apparent topo IV decatenation paradox is finally solved, it seems that during replication all the possible topological forms RIs can adopt could have some role. The topological cycle of a replicon appears to involve supercoiling, precatenation, knotting, catenation and decatenation (see Fig 3). Whether or not the changes that have been observed for small plasmids also apply to the large topological domains of bacteria and linear eukaryotic chromosomes remains to be shown.

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